# Serum Cotinine Level as Predictor of Lung Cancer Risk

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## Abstract

Background: **No prospective studies are available on serum cotinine level as a marker of lung cancer risk.**
Methods: **We analyzed serum cotinine level among 1,741 individuals enrolled since the 1970s in a prospective study of Norwegian volunteers who developed lung cancer during the follow-up and 1,741 matched controls free from lung cancer. Serum cotinine was measured with a competitive immunoassay. Regression dilution was corrected for based on repeated measures on samples from 747 subjects.**
Results: **Mean serum cotinine level was higher in cases than in controls. Compared with subjects with a cotinine level of ≤5 ng/mL, the odds ratio of lung cancer was increasing linearly, reaching 55.1 (95% confidence interval, 35.7-85.0) among individuals with a serum cotinine level of >378 ng/mL. There was no clear suggestion of a plateau in risk at high exposure levels. Odds ratios were very similar in men and women. We found no association between serum cotinine level (range, 0.1-9.9 ng/mL) and lung cancer risk among self-reported nonsmokers and long-term quitters (79 cases and 350 controls).**
Discussion: **The association between tobacco smoking and lung cancer risk might be stronger than is estimated from questionnaire-based studies. Serum cotinine level is a predictor of risk of lung cancer among smokers. The reported plateau in risk at high doses is likely due mainly to artifacts. There is no difference between men and women in the carcinogenicity of tobacco smoking.** (Cancer Epidemiol Biomarkers Prev 2006;15(6):1184–8)

## Introduction

Tobacco smoking is the main cause of lung cancer in most human populations. The strength of the association and the proportion of cases of lung cancer attributable to smoking vary across populations, based on past prevalence of smoking and detailed aspects of the habit, such as type of tobacco products and inhalation patterns (1). The strongest direct evidence of the carcinogenic effect of tobacco smoking on the human lung comes from epidemiologic studies in which the assessment of exposure was based on self-reported information on personal smoking habit, typically collected via questionnaire. Quantitative estimates of the risk of lung cancer following tobacco smoking have elucidated several important aspects of the carcinogenicity of tobacco, in particular, a stronger carcinogenic effect of duration of smoking compared with dose rate (2, 3). They have also suggested a plateau in the increasing risk above 30 cigarettes per day (4), whereas a higher risk among women compared with men given the same level of exposure has been debated (5-7). Questionnaire-based self-reported information on tobacco smoking is considered more valid than that on other lifestyle habits and environmental exposures (8), and validation studies have shown that self-reported information on smoking status is confirmed by biomarker-based assessments (9-12). Similarly, the validity of self-reported current amount of smoking was positively correlated with serum cotinine level in at least six studies including >100 subjects (13-18).

Cotinine is the main metabolite of nicotine, and its serum or plasma level is a useful marker of tobacco smoking (9, 19). Furthermore, the use of serum cotinine rather than questionnaire data to measure tobacco exposure integrates different aspects of the exposure, including tobacco composition, uptake, distribution, and individual differences in metabolism (9). Therefore, an analysis of the relationship between serum cotinine and lung cancer risk might contribute to a better understanding of the quantitative aspects of tobacco-related lung carcinogenesis in humans (20).

The Janus serum bank offers the opportunity to investigate for the first time prospectively the association between lung cancer risk and serum level of cotinine. The bank comprises 360,897 serum samples collected from 286,579 persons in Norway from 1973 onwards who can be followed up for cancer incidence. Smoking status is known for 89% of cohort members. We specifically addressed the following questions: (*a*) the strength of the association between tobacco smoking, measured via serum cotinine, and lung cancer risk; (*b*) the presence of a plateau in the risk function at high doses; (*c*) the presence of a difference in risk between men and women tested with gender-specific analyses.

## Materials and Methods

The Janus serum databank includes frozen serum samples from blood donors and from participants in different health surveys conducted in Norway since 1973 (21). At the time of blood collection, the individuals gave an informed consent to participate in the study and filled in a questionnaire on various aspects of their lifestyle, including tobacco smoking. The questions on tobacco smoking varied by time and place, but all individuals were requested to report whether they were current, former, or never smokers of any tobacco product (cigarettes, pipe, or cigars), although the definition of former smokers was not fully consistent. Regular smokers were defined as individuals smoking at least one cigarette, one cigar, or one pipe a day; information on occasional smoking was also collected. Information on duration of smoking and duration of quitting was limited; no information on exposure

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P. Boffetta and A. Andersen had full access to all the data and take joint responsibility for the integrity of the data and the accuracy of the data analysis.

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to involuntary smoking was available for members of the cohort.

Following approval of relevant ethical committees, we linked the Janus serum databank to the national mortality and emigration registries maintained by the Statistics Norway, from which information on date of death or emigration was obtained. We further linked it with the national cancer registry, which provided information on cancer incidence. For linkages between different sources, we used the unique social security number assigned to each citizen in Norway. We excluded the first year of follow-up after sample collection. As a result of the linkages, we identified, during an average of 11.5 years of follow-up, incident cases of lung cancer. For each case, we randomly selected one control among individuals who fulfilled the matching criteria and were alive and free from lung cancer at the date of diagnosis of the case. Matching criteria were sex, year of birth (±2 years), time of enrollment (±1 year), and geographic region of enrollment.

We retrieved the serum samples of the cases and controls from the bank and labeled them with a dummy number to guarantee blindness as to case-control status. We measured cotinine using a qualitative immunoassay method (OraSure Technologies, Inc., Bethlehem, PA), which is run as a quantitative assay and is based on the competition between free cotinine in the sample and cotinine bound to horseradish peroxidase, for an antibody fixed onto a polystyrene microplate. Following incubation, excess enzyme is washed away; substrate is added; and the measured absorbance is inversely proportional to the amount of free cotinine in the sample. We modified the standard calibrators of the assay (at level 0, 10, 25, and 50 ng/mL) by including additional calibrators at 100, 250, and 500 ng/mL (which were supplied by OraSure Technologies), to extend the standard curve and make the assay more suitable for higher levels of smoking.. In a separate validation exercise, samples were also tested with gas chromatography (22): across the range of 0 to 20 ng/mL of cotinine, there was a 95% correlation between the results of the two methods.

We tested differences in average log-transformed serum cotinine levels of cases and controls based on unbalanced ANOVA and calculated odds ratios (OR) of lung cancer and their 95% confidence intervals (95% CI) for eight categories of increasing serum cotinine level among controls, using individuals with a level of ≤5 ng/mL as the reference category, based on conditional logistic regression analysis. Such an analysis adjusts for the potential confounding effect of matching variables. Additional CIs were derived by first calculating floating absolute risks for the variance of the logarithm of relative risk and subsequently incorporating a Taylor series expansion (23). The floating absolute risk method avoids the problem of correlation of regression variables to the baseline (unexposed) category, resulting in inflated SEs.

Error in the cotinine measurements due to dilution regression bias (24) was assessed by measuring in the same laboratory and with the same method cotinine in repeated samples. For 747 of the individuals included in the study, two different serum samples were available, which had been acquired on average 7.6 years apart. The correlation coefficient between the first and the second sample was 0.774 and did not differ systematically by cotinine level: this factor was then used to adjust the ORs. The statistical analyses were conducted using the STATA and SAS packages.

We also conducted stratified analyses according to gender, age, year of enrollment in to the cohort, and duration of follow-up. The main analysis was based on the results from the first available serum sample; additional analyses were conducted based on average cotinine level when multiple samples were available. Finally, an analysis was restricted to 79 cases and 350 controls who (*a*) reported either not having smoked at the time of enrollment or earlier (never smokers) or having quit smoking >5 years before enrollment (long-term quitters) and (*b*) had a serum cotinine level below 10 ng/mL, to address the question of whether serum cotinine level is a marker of exposure to involuntary smoking. In this analysis, we used three categories of serum cotinine level based on the distribution among controls. We selected a threshold of 10 ng/mL to obtain a more specific definition of nonsmokers, but we conducted sensitivity analyses based on other thresholds.

## Results

A total of 1,741 pairs of one case and one control each were included in the analysis. Their distribution by self-reported smoking status, based on questionnaire responses, and selected demographic characteristics are presented in Table 1. Men comprised 76% of the study population. The majority of study subjects were recruited into the study when they were below 45 years of age; the average duration of follow-up among cases was 14.2 years. A total of 49 cases and 31 controls were ever smokers of cigar or pipe. Compared with never smokers, the OR of lung cancer was 3.09 (95% CI, 2.05-4.66) for ex smokers, 18.3 (95% CI, 12.6-26.7) for current smokers, and 13.8 (95% CI, 8.29-23.0) for ever smokers. The mean serum cotinine level was 286.4 ng/mL among cases and 125.8 ng/mL among controls ($P_{difference} < 0.0001$). Significant differences in mean serum cotinine level were seen in all smoking groups, except never smokers (Table 2).

The results of the main analysis of serum cotinine level, disregarding the self-reported smoking status, and lung cancer

**Table 1. Distribution of cases and controls by sex, age at first sample, year of first sample, duration of follow-up, attained age, and smoking status**

| | Cases (*N* = 1,741), *n* (%) | Controls (*N* = 1741), *n* (%) |
|---|---|---|
| Sex* | | |
| Men | 1,322 (75.9) | 1,322 |
| Women | 419 (24.1) | 419 |
| Age at first sample (y)* | | |
| <45 | 980 (56.3) | 1,019 (58.5) |
| 45-54 | 617 (35.4) | 599 (34.4) |
| 55-64 | 44 (2.6) | 22 (1.3) |
| ≥65 | 100 (5.7) | 101 (5.8) |
| Year of first sample* | | |
| 1970-1975 | 779 (44.8) | 835 (48.0) |
| 1976-1980 | 292 (16.8) | 319 (18.3) |
| 1981-1985 | 95 (5.5) | 69 (4.0) |
| 1986-1990 | 535 (30.7) | 476 (27.3) |
| 1991-1995 | 40 (2.2) | 42 (2.4) |
| Duration of follow-up (y)* | | |
| 1-5 | 191 (11.0) | 185 (10.6) |
| 5-9 | 391 (22.5) | 356 (20.4) |
| 10-14 | 376 (21.6) | 351 (20.2) |
| 15-19 | 317 (18.2) | 342 (19.6) |
| 20-24 | 358 (20.6) | 393 (22.6) |
| 25-29 | 108 (6.2) | 114 (6.6) |
| Attained age (y) † | | |
| <45 | 64 (3.7) | 64 (3.7) |
| 45-54 | 551 (31.6) | 564 (32.4) |
| 55-64 | 557 (32.0) | 550 (31.6) |
| ≥65 | 569 (32.7) | 563 (32.3) |
| Smoking status | | |
| Never smoker | 53 (3.0) | 445 (25.6) |
| Ex smoker | 128 (7.4) | 411 (23.6) |
| Current smoker | 1,393 (80.0) | 727 (41.8) |
| Ever smoker ‡ | 96 (5.5) | 67 (3.8) |
| Missing | 71 (4.1) | 91 (5.2) |

*The distributions of cases and controls are similar as result of individual matching.

†$P_{difference}$ between cases and controls ($\chi^2$ square test) < 0.0001.

‡Current or ex smoker (only information on ever/never smoking status was available in a subset of study subjects).

**Table 2. Mean serum cotinine level (ng/mL) in cases and controls by sex, attained age, duration of follow-up, and smoking status**

| | Cases | Controls | $F$* | $P > F$ |
|---|---|---|---|---|
| Sex | | | | |
| Men | 293.9 | 134.8 | 762.8 | <0.0001 |
| Women | 262.8 | 97.2 | 275.7 | <0.0001 |
| Attained age (y) | | | | |
| ≤60 | 296.5 | 121.4 | 589.1 | <0.0001 |
| >60 | 275.9 | 130.3 | 430.8 | <0.0001 |
| Duration of follow-up (y) | | | | |
| <10 | 274.1 | 112.1 | 320.8 | <0.0001 |
| ≥10 | 292.6 | 131.9 | 704.6 | <0.0001 |
| Smoking status | | | | |
| Never smoker | 10.7 | 11.5 | 0.64 | 0.43 |
| Ex smoker | 95.7 | 22.1 | 11.2 | 0.003 |
| Current smoker | 311.4 | 247.8 | 84.0 | <0.0001 |
| Ever smoker | 338.0 | 264.6 | 6.2 | 0.04 |
| Missing | 276.8 | 75.6 | 67.1 | <0.0001 |

*Unbalanced ANOVA.

risk are reported in Table 3. Compared with subjects with a cotinine level of ≤5 ng/mL, the OR of lung cancer was 0.93 (95% CI, 0.46-1.90) in the category 5.1 to 24.7 ng/mL of serum cotinine and increased linearly above that level up to a value of 55.1 (95% CI, 35.7-85.0) for a cotinine level of >378.8 ng/mL. The CI of the reference category based on floating absolute risks was 0.74 to 1.36; the corresponding interval in the category with the highest cotinine level was 41.1 to 73.8. Results were remarkably similar in men and women, whereas the ORs were higher among young subjects than among older ones ($P_{\text{interaction}}$ = 0.02; Table 4). Results were also similar among subjects enrolled in the cohort before 1982 and among subjects enrolled in 1982 or later.

When we repeated the analysis after exclusion of ex smokers, the OR of lung cancer in the category 5.1 to 24.7 ng/mL of serum cotinine was 0.47 (95% CI, 0.11-2.00), and the ORs for serum cotinine above that level were higher than the corresponding values, including ex smokers (Fig. 1).

The results of the logistic regression analysis among nonsmokers (uncorrected for regression dilution) are reported in Table 5: no clear association was suggested between serum cotinine level and lung cancer risk; however, the OR for >2.3 ng/mL serum cotinine in subjects aged <55 years was 1.64 (95% CI, 0.47-5.72). The CI of the reference category based on floating absolute risks was (95% CI, 0.58-1.71); corresponding intervals for the other two tertiles of the distribution were 1.02 to 2.41 and 0.67 to 1.67. Results were similar in men and women. Correction for regression dilution, based on repeated samples among 87 nonsmoking cases and controls, resulted in risk estimates very close to those reported in Table 5 (not shown in detail). The results shown in Table 5 were not sensitive to the choice of the cotinine level (10 ng/mL) used as cut point: the OR for the upper tertile of the distribution was 0.97 (95% CI, 0.51-1.83) when no threshold was applied (88 cases; range, 0.1-37.8 ng/mL and 401 controls; range, 0.1-474.9 ng/mL); it was 0.90 (95% CI, 0.47-1.72) with cut point at 25 ng/mL of cotinine (81 cases and 375 controls) and 1.36 (95% CI, 0.64-2.91) with cut point at 5 ng/mL of cotinine (74 cases and 297 controls).

## Discussion

The results of this study show a strong, linear dose-response relationship between serum cotinine level and lung cancer risk among smokers. Measurement of serum cotinine, in particular if based on repeated samples, would reduce exposure misclassification and improve the estimate of the carcinogenic effect of smoking. In our study, serum cotinine level, when measured in a single sample taken several years before onset of cancer, was a strong predictor of risk, suggesting that this approach is more relevant than traditional (e.g., questionnaire-based methods to measure exposure to tobacco smoke and to investigate its carcinogenic effects).

One important result of our analysis is the lack of a clear plateau in the relative risk of individuals with high serum cotinine levels. This is at odds with the observation of such an effect with amount of cigarettes smoked per day (e.g., based on the results of a pooled analysis of case control studies; ref. 4) and suggests that assessment of tobacco smoking using a questionnaire might be particularly problematic at high doses. The observed shape of the dose-response relationship based on questionnaire data would therefore be mainly explained by artifacts, such as misclassification at high doses or reduced inhalation of heavy smokers (18, 25), rather than reflecting a true biological phenomenon, possibly linked to saturation of enzymatic pathways. A similar conclusion of possible misclassification at high doses of self-reported cigarette smoking was reached in an analysis comparing self-reports with plasma cotinine level in a population of 32,000 controls included in a study of myocardial infarction from the United Kingdom (17). In our study, only limited information was available on amount and duration of tobacco smoked by study subjects, preempting detailed analyses based on the comparison of serum cotinine and self-reported tobacco smoking.

No previous prospective study analyzed lung cancer risk according to serum cotinine level. In a prospective study from Scotland, the incidence of coronary heart disease was associated with serum cotinine level, and the dose-response was comparable with that found with self-reported smoking (26, 27).

The ORs estimated in men and women were remarkably similar. These results do not support the hypothesis of a higher susceptibility to lung cancer of women compared with men, which has been proposed based on evidence from epidemiologic and toxicologic studies (5, 28, 29). Indeed, several studies that carefully quantified tobacco exposure provided evidence of a comparable increase in lung cancer risk in the two sexes (3, 30, 31), suggesting that epidemiologic studies providing evidence for a stronger risk among women might have resulted either from differential exposure misclassification among sexes or from aspects of tobacco smoking (e.g., inhalation), which are not adequately assessed in questionnaire-based studies.

The apparent stronger association detected in young people (Table 4) might be explained by the lower proportion of ex smokers in this group compared with older people. An analysis restricted to never and current smokers resulted in similar risk estimates in the two groups (data not shown in detail).

**Table 3. ORs of lung cancer for serum cotinine level**

| Serum cotinine level (ng/mL)* | $N$ cases | $N$ controls | OR (95% CI) |
|---|---|---|---|
| 0.1-5.0 (reference) | 135 | 755 | 1.00 |
| 5.1-24.7 | 21 | 124 | 0.93 (0.46-1.90) |
| 24.8-114.7 | 51 | 123 | 2.80 (1.55-5.05) |
| 114.8-180.2 | 118 | 123 | 7.67 (4.75-12.4) |
| 180.3-223.8 | 157 | 123 | 13.8 (8.70-22.0) |
| 223.9-271.9 | 235 | 124 | 24.9 (15.9-39.0) |
| 272.0-317.7 | 274 | 123 | 30.4 (19.5-47.3) |
| 317.8-378.8 | 308 | 123 | 33.1 (21.3-51.4) |
| >378.8 | 442 | 123 | 55.1 (35.7-85.0) |

NOTE: OR is based on conditional logistic regression analysis, adjusting for sex, year of birth, time of enrollment, and geographical region.
*Categories are based on serum cotinine level only, not on self-reported smoking status.

**Table 4. OR of lung cancer for serum cotinine level stratified by sex and attained age**

| Serum cotinine level* (ng/mL) | *N* cases | *N* controls | OR (95% CI) | *N* cases | *N* controls | OR (95% CI) |
|---|---|---|---|---|---|---|
| Sex | Women | | | Men | | |
| 0.1-5.0 (reference) | 47 | 207 | 1.00 | 88 | 548 | 1.00 |
| 5.1-114.7 | 25 | 75 | 1.54 (0.65-3.69) | 47 | 172 | 1.83 (1.02-3.30) |
| 114.8-223.8 | 72 | 48 | 11.9 (5.56-25.3) | 203 | 198 | 10.5 (6.59-16.6) |
| 223.9-317.7 | 124 | 48 | 23.1 (11.0-48.3) | 385 | 199 | 27.8 (17.7-43.8) |
| >317.7 | 151 | 41 | 47.2 (21.3-105) | 599 | 205 | 42.1 (27.1-65.3) |
| Attained age | ≤60 y | | | >60 y | | |
| 0.1-5.0 (reference) | 59 | 388 | 1.00 | 69 | 345 | 1.00 |
| 5.1-114.7 | 30 | 116 | 1.60 (0.73-3.51) | 39 | 103 | 1.96 (1.01-3.78) |
| 114.8-223.8 | 118 | 110 | 13.1 (7.17-24.1) | 150 | 123 | 10.1 (5.85-17.3) |
| 223.9-317.7 | 248 | 121 | 29.9 (16.7-53.5) | 238 | 115 | 25.3 (14.6-43.9) |
| >317.7 | 393 | 103 | 60.0 (33.6-107) | 318 | 118 | 33.7 (19.6-57.9) |

NOTE: OR is based on conditional logistic regression analysis, adjusting for year of birth, time of enrollment, geographical region, and in the analysis of attained age and sex.
*Categories are based on serum cotinine level only, not on self-reported smoking status.

We found no appreciable effect of serum cotinine level as a marker of involuntary smoking among nonsmokers. Serum cotinine levels are higher in our study (mean, 2.2 ng/mL) compared with other studies of nonsmokers (e.g., 1.4 ng/mL in a study from the United States; ref. 32). This difference might result from a higher exposure to involuntary smoking experienced by members of the Janus population, but it can also be due to higher sensitivity and precision of the cotinine assay used in our study, or to a higher proportion of misclassification of smokers. However, the prospective nature of the study makes it difficult to envisage that misclassification of exposure would occur differently among cases compared with controls. Use of smokeless tobacco products, which is more prevalent in Norway compared with countries, such as the United Kingdom and the United States (e.g., 1985 prevalence of daily or occasional snuff use in men ages 15 to 75 years was 7%, with little difference between age groups, unpublished data, Statistics Norway, 2003), might also have contributed to the elevated cotinine level of nonsmokers as shown among Swedish users of smokeless tobacco (33). Consistent with the limited sensitivity of a single serum cotinine measurement to detect any carcinogenic effect of involuntary tobacco smoking was the lack of an increased risk among subjects with serum cotinine level in the range typical of heavy exposure to involuntary smoking as well as of weak active smoking (ref. 34; Table 3). If serum cotinine is considered a marker of exposure to environmental tobacco smoke, the results of our study are not consistent with the evidence from questionnaire-based studies of a carcinogenic effect of this agent on the human lung (1); however, they are consistent with the results of a European study including 59 never-smoking cases of lung cancer (35). Although low statistical power is the most plausible explanation of the negative results among nonsmokers (our study had 80% power to detect an OR of ≥1.9 for cotinine level above the median among controls, a value which is greater than those found in questionnaire-based studies), an additional explanation might be misclassification of exposure due to the use of a single sample, which was taken on average 11.5 years before diagnosis, in particular because environmental tobacco smoke exposure is often intermittent, resulting in greater variability in serum cotinine level than in active smokers.

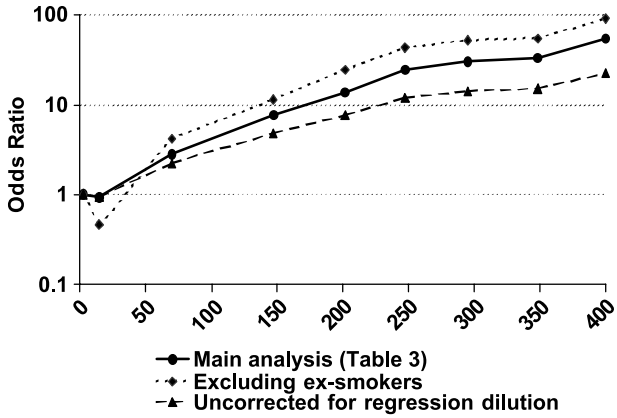


**Figure 1.** OR of lung cancer risk for serum cotinine level.

Degradation of cotinine during long-term storage of samples should not have affected our results. Cotinine levels remain stable in frozen serum samples, as it has been shown in studies comparing repeated measurements on the same samples (36, 37). Furthermore, cases and controls were matched for year of enrollment in the study, and degradation of cotinine from the samples would have affected equally the two groups.

In conclusion, our study contributes to an understanding of the association between a biomarker of tobacco smoke exposure and lung cancer among smokers. The strength of the association may be underestimated in studies that do not correct for misclassification of exposure. No plateau is apparent in the risk of lung cancer at high doses. Tobacco smoking seems to exert a comparable carcinogenic effect in men and women.

**Table 5. Results of conditional logistic regression analysis of serum cotinine level among never smokers**

| | *N* cases | *N* controls | OR (95% CI) |
|---|---|---|---|
| Overall (ng/mL) | | | |
| <1.1 (reference) | 23 | 116 | 1.00 |
| 1.1-2.3 | 33 | 113 | 1.57 (0.79-3.13) |
| 2.4-9.9 | 23 | 121 | 1.06 (0.52-2.14) |
| Men (ng/mL) | | | |
| <1.1 (reference) | 10 | 67 | 1.00 |
| 1.1-2.3 | 20 | 87 | 1.58 (0.61-4.12) |
| 2.4-9.9 | 17 | 89 | 1.25 (0.51-3.09) |
| Women (ng/mL) | | | |
| <1.1 (reference) | 13 | 49 | 1.00 |
| 1.1-2.3 | 13 | 26 | 1.61 (0.59-4.36) |
| 2.4-9.9 | 6 | 32 | 0.74 (0.22-2.43) |
| Age < 55 (ng/mL) | | | |
| <1.1 (reference) | 7 | 37 | 1.00 |
| 1.1-2.3 | 11 | 27 | 2.80 (0.79-9.85) |
| 2.4-9.9 | 8 | 35 | 1.64 (0.47-5.72) |
| Age ≥ 55 (ng/mL) | | | |
| <1.1 (reference) | 16 | 79 | 1.00 |
| 1.1-2.3 | 22 | 86 | 1.20 (0.53-2.74) |
| 2.4-9.9 | 15 | 86 | 0.86 (0.37-2.03) |

NOTE: Self-reported never smokers and long-term quitters with serum cotinine level below 10 ng/mL were included in the analysis (79 cases and 350 controls).

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